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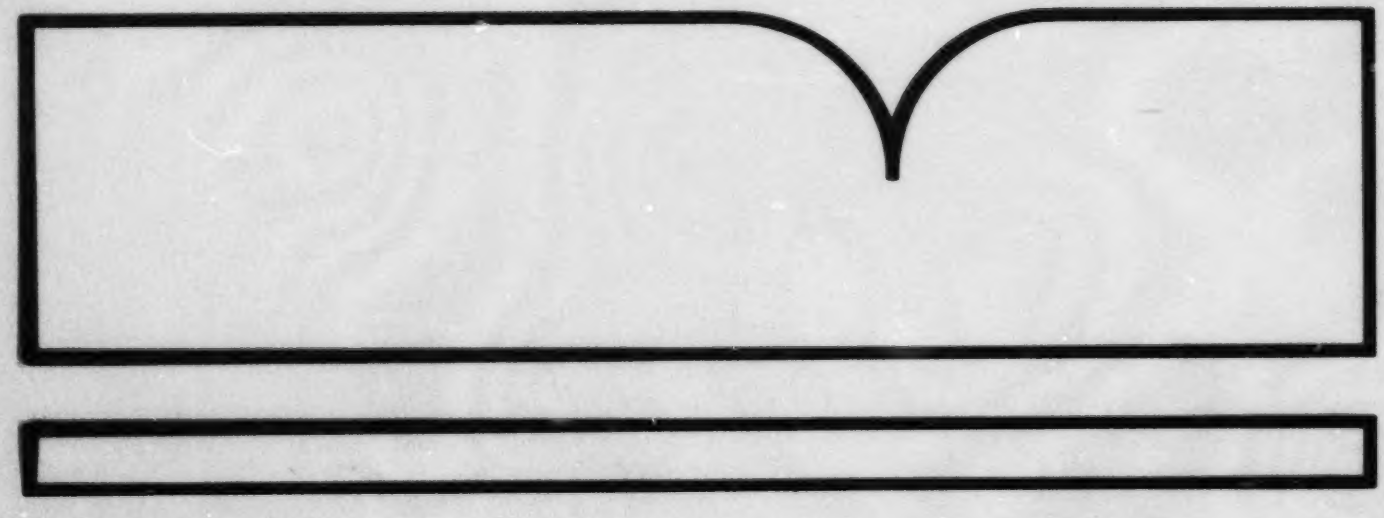

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16. ABSTRACT

A committee of scientists was established by the U.S. Environmental Protection Agency to appraise the current state of aneuploidy test methodology, to compile and analyze published data on the chemical induction of aneuploidy, and to provide guidance for additional test development and validation. The committee was organized into 9 working groups, representing different eukaryotic organisms ranging from fungi to whole mammals. The Environmental Mutagen, Carcinogen, and Teratogen Information Program at Oak Ridge National Laboratory identified and provided literature to the working groups. The 9 reports were prepared by the individual working groups. The reports include: (1) descriptions of the available testing approaches and general principles to be followed for protocol development, (2) criteria for evaluating data, and (3) recommendations for future test development and basic research. The evaluated results are presented in tables. For the positive and negative calls, the lowest effective dose tested or the highest ineffective dose tested, respectively, and the range of concentrations tested are provided. An overall summary report was prepared that summarizes the conclusions and recommendations of the 9 working groups and the chemical data base. It is hoped that these reports will provide a framework for further test development and validation of aneuploidy assays.

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Detection of chemically induced aneuploidy with plant test systems *

Shahbeg S. Sandhu [1], Baldev K. Vig [2] and Milton J. Constantin [3]

[1] *Genetic Toxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711,*
[2] *Department of Biology, University of Nevada, Reno, NV 89557, and*
[3] *Phyton Technologies, Inc., Knoxville, TN 37931 (U.S.A.)*

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Summary

The utility of plant test systems for detecting chemically induced aneuploidy was evaluated by using papers published in peer-reviewed journals. A total of 147 papers were provided to the group by the Environmental Mutagen Information Center. Based on the criteria established by the Gene-Tox Committee (Waters and Auletta, 1981), 22 papers were selected for in-depth review. Only those papers listing additional, missing, or lagging chromosomes in the meiotic or mitotic cells were included in this review. Although most plant test systems may be developed to utilize either mitotic or meiotic cells for cytogenetic analysis, only a few have been employed for this purpose. In this review, *Allium cepa* was found to be the most commonly used test system. Other species used less frequently were *Vicia faba, Hordeum vulgare,*

* Report of the Aneuploidy Data Review Committee (Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC).

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Sorgham vulgare, and *Pennisetum americanum.* None of the plant test systems have been sufficiently utilized to warrant judgment for its sensitivity and specificity for detecting induced aneuploidy. A suggested protocol for detecting chromosomal malsegregation in meiotic or mitotic cells is presented. Further development and utilization of plant tissue culture techniques and morphological markers identifiable in the seedling stages is recommended for detecting chemically induced aneuploidy.

(1) Introduction

Aneuploidy in plants was first reported by Lutz (1909) in two plants from a cross between *Oenothera lata* and *O. gigas.* Blakeslee (1921a, b, c) laid the real foundation for the study of aneuploidy in plants with his studies on jimson weed, *Datura stramonium.* He coined the term trisomy to describe the addition of one chromosome to the basic complement and isolated 12 trisomics in Datura, one for each pair of chromosomes. After Blakeslee's *D. stramonium* studies, aneuploids were reported in most major plant species (see review by Khush, 1973).

Aneuploidy occurs spontaneously in natural plant populations and has been induced by treatment with chemical and physical agents. The possibility that chemical and physical agents induce aneuploidy and its associated health hazards in humans has stimulated new interest in the subject. The role of aneuploidy in initiating carcinogenesis has not been established, but it has been suggested that genomic alterations may predispose some individuals to cancer (Arlett and Lehmann, 1978; Levan and Mitelman, 1977; Bloom, 1972; Tsutsui et al., 1983). Aneuploidy's importance in the field of chemical mutagenesis has been emphasized only recently and a limited amount of work on this subject has been reported (Zimmermann et al., 1979). The biological systems most commonly used to detect chemically induced aneuploidy are fungi (de Bertoldi et al., 1980), yeast (Parry et al., 1981), and insects (Liang et al., 1983; Zimmering, 1982).

Plants offer unique advantages as test systems for evaluating the potential of chemicals to induce aneuploidy. Plant systems are less expensive and less time consuming than any mammalian system (Grant et al., 1981). Somatic regeneration and continuous propagation of certain karyotypes of interest provide other advantages. Chromosome analysis of male gametophytes (pollen mother cells (PMC)) (Wuu and Grant, 1967; Ahmed and Grant, 1972) is easy; an additional advantage in time and cost. The usefulness of a few plant test systems in the evaluation of chromosomal aberrations in general has been reviewed under the Gene-Tox Program (Ma, 1982a, b; Constantin and Nilan, 1982; Constantin and Owens, 1982; Grant, 1982a). These reviewers grouped several types of chromosomal aberrations into one category and did not report induced numerical chromosomal aberrations as a separate category. This report attempts to assess the available information and to determine the usefulness of plant test systems in the detection of chemically induced chromosomal imbalance.

(2) Selection criteria

22 publications of a total of 147 provided by the Environmental Mutagen Information center were selected for in-depth review based on criteria developed by the Gene-Tox Committee (Waters and Auletta, 1981). Gene-Tox criteria include use of concurrent positive and negative controls, evaluation of one chemical at a time, and a clear statement of treatment conditions (e.g., solvents used, pH of chemical solutions, length of recovery period, and growth conditions), and number of cells analyzed. Only those reports describing addition or deletion of an entire chromosome or a lagging chromosome were included in the present review. More frequently than not, relaxed criteria were applied in accepting the articles for the present review. For example, one of the selection criteria was that the test protocol must include concurrent positive and negative controls. Few papers, if any, included positive controls. The difficulty may be that no compounds are known to cause aneuploidy in a particular plant test system. Some compounds (e.g., colchicine and related alkaloids) cause chromosomal doubling in most plant species and aneuploidy in some species, but their

TABLE 1

EPA ANEUPLOIDY DATA FOR PLANTS

Chemical	Cas No.	Assay	Call [a]	LEDT/HIDT [b]	Range	Reference
Benzo[*a*]pyrene	50-32-8	*A. cepa* roots	–	10 μg/ml	0.5–10 μg/ml	Fiskesjö (1981)
Aspirin	50-78-2	*A. cepa* roots	+	0.05%	0.01–0.10%	Shanthamurthy and Rangaswamy (1979)
Chloramphenicol	56-75-7	*H. vulgare* roots	+	300 μg/ml	300 μg/ml	Yoshida et al. (1972)
Progesterone	57-83-0	*P. sativum* roots	I		Saturated solution	Prasad and Haider (1982)
Tetracycline	60-54-8	*A. cepa* roots	+	16 μg/ml	12–20 μg/ml	Mann (1978)
Ethyl methanesulfonate	62-50-0	*P. americanum* PMC	+	2000 μg/ml	2000–4000 μg/ml	Laxmi et al. (1975)
Colchicine	64-86-8	*A. cepa* roots	+	0.1% × 3 h	0.1–0.5%	Davidson et al. (1983)
		P. vulgaris progeny	I		0.1%	Mok and Mok (1977)
		C. capillaris (diploid) roots	+	5% × 4 h	5%	Pltonova and Svarinskaya (1980)
		C. capillaris (tetraploid) roots	+	0.5%	0.5%	Platonova and Scarinskaya (1980)
		C. capillaris roots	+	0.5%	0.5%	Sakharov et al. (1969)
		H. vulgare roots	–	0.1%	0.1%	Schwammenhöferova and Ondrej (1978)
Cycloheximide	66-81-9	*A. sativum* roots	W+	1.42 mM	1.42 mM	Wagenaar (1983)
MNNG	70-25-7	*A. cepa* roots	I		0.5–10.0 μg/ml	Fiskesjö (1981)
Methoxyflurane	76-38-0	*V. faba* roots	I		0.2–0.8%	Grant et al. (1977)
2,4,5-Trichlorophenoxyacetic acid	93-76-5	*A. cepa* roots	W+	0.01 ppm	0.001–1.0 ppm	Fiskesjö et al. (1981)
4-Chloro-*o*-tolyloxyacetic acid	94-74-6	*A. cepa* roots	–	1.0 ppm	0.05–1.0 ppm	Fiskesjö et al. (1981)
2,4-Dichlorophenoxyacetic acid	94-75-7	*A. cepa* roots	W+	1.0 ppm	0.001–1.0 ppm	Fiskesjö et al. (1981)
2,4,5-Trichlorophenol	95-95-4	*A. cepa* roots	W+	1 ppm	0.5–50 ppm	Fiskesjö et al. (1981)
2,4-Dichlorophenol	120-83-2	*A. cepa* roots	–	50 ppm	0.5–50 ppm	Fiskesjö et al. (1981)
Griseofulvin	126-07-8	*M. sativa* suspension culture	+	150 mg/ml	150 mg/ml	Lo Schiavo et al. (1980)
Halothane	151-67-7	*V. faba* roots	I		1–4%	Grant et al. (1977)
Diethyl pyrocarbonate	1609-47-8	*H. vulgare* roots	+	0.6%	0.3–0.6%	Natarajan et al. (1966)
Atrazine	1912-24-9	*S. vulgare* PMC	+	2.7 kg/ha	2.7 kg/ha	Liang et al. (1967)
		S. vulgare PMC	+	4.8 lb/acre	2–4.8 lb/acre	Liang et al. (1969)
N-Furfuryladenosine	4338-47-0	*L. esculentum* callus tissue	I		1–10 mg/l	Koforak et al. (1977)
Nitrous oxide	10024-97-2	*H. vulgare* roots	I		4 atm/l	Dvorak et al. (1973)
		T. aestivum embryos	I		4 atm/l	Dvorak et al. (1973)

TABLE 1 (continued)

Chemical	Cas No.	Assay	Call [a]	LEDT/HIDT [b]	Range	Reference
Platinum diamino-dinitrodichloride	18726-50-6	*P. americanum* PMC	I		0.5–50 μM	Dhesi et al. (1983)
Adriamycin	23214-92-8	*A. cepa* roots	I		0.1–10.0%	Mercykutty and Stephen (1980)
Sodium azide	26628-22-8	*P. vulgaris* leaves	+	0.001 μg/ml	0.001–0.002 μg/ml	Vig (1973)

[a] +, positive; W+, weak positive; −, negative; I, inconclusive.
[b] LEDT, lowest effective dose tested; HIDT, highest ineffective dose tested.

ability to cause chromosomal malsegregation is not ubiquitous. The requirement for a negative control was also ignored if multiple treatment doses were applied and genetic response was clearly concentration related.

None of the reviewed papers applied rigorous statistical analysis; therefore, we had to use subjective judgment in evaluating the genetic response data. All papers were independently evaluated by 3 reviewers. A summary of the reviewers' comments is presented in Table 1.

(3) Test systems

The plant cytogenetic systems that were reviewed used mitotic tissues (meristematic root or leaf cells), meiotic cells (PMC), and somatic cells in culture. Most plant cytogenetic test systems can be developed to use both meiotic and mitotic cells; only a few actually have used both types of cells.

With rare exceptions, the plant test systems were not specifically developed to detect aneuploidy, but to increase genetic variability that could be used to improve crops. These assays gained popularity because of their usefulness in evaluating the ability of chemical and physical agents to induce cytogenetic damage, primarily chromosomal structural changes. Data on aneuploidy were collected as ancillary observations. However, these cytogenetic systems can be used, with practically no modification, to detect the induction of aneuploidy by environmental pollutants.

Of the plant systems reviewed, the most frequently used was the *Allium cepa* root cell assay. *A. cepa* has a relatively small number of large chromosomes that makes detection of chromosomal aberrations easier. *Pennisetum americanum, Hordeum vulgare,* and *Sorghum vulgare* were also used, although the number of chemicals tested in each of these species is very small.

(4) Test protocols

The generalized test protocols for studying malsegregation in mitotic tissues (primarily root tips and leaves) and in meiotic cells (PMC) will be described in the present review. For a more detailed discussion of these protocols, see Grant (1982a, b, for *A. cepa*); Constantin and Nilan (1982, for *H. vulgare*); Ma (1982a, b, for *Vicia faba* and Tradescantia); and Vig (1975, for *Glycine max*). The protocols for *P. americanum* and *S. vulgare* are similar to those described for *H. vulgare* (Constantin and Nilan, 1982).

(4.1) Mitotic cells

(4.1.1) Root tip assay. Detailed protocols for the detection of induced chromosome aberrations in mitotic tissue have been published by Ma (1982a, b), Grant (1982a, b), and Constantin and Nilan (1982). Any plant species with a relatively small number of large chromosomes may be used for detecting aneuploidy; those used most often are *A. cepa, V. faba,* and *H. vulgare.* The salient features of the protocol for these species are given here. Seeds of Vicia or Hordeum, selected for uniformity, may be surface sterilized by soaking for 20 min in 1–5% calcium hypochlorite to eliminate pathogens from the seed surface, and

then washed in running water. Seed of Vicia or Hordeum are grown in petri plates, and treatment may be carried out when roots are 2–3 cm long. For Allium, young, uniformly sized (10–30 g) bulbs are denuded by removing the loose outer scales and scraped so that the apices of the root primordia are exposed. Bulbs are submerged in tap water in bottles or tanks to about one-quarter the depth of the bulb. Water should be changed daily and continuously aerated by bubbling, and the containers maintained in the dark at 20°C. Bulbs with 1–5-cm root tips are transferred to a similar vessel containing freshly prepared, pH-controlled test solution.

After treatment, excised roots are fixed in 95% alcohol–glacial acetic acid (3 : 1) for at least 30 min and subsequently may be kept in 70% alcohol in the refrigerator. Aceto-carmine is adequate for staining the roots. Better results may be obtained by first hydrolyzing the excised roots in 1 N HCl at 60°C for 7 min and then staining in Feulgen reagent.

A simple squash method is used to prepare temporary slides. Semi-permanent slides may be prepared by using the dry ice (Conger and Fairchild, 1953) or quick-freezing (Sharma and Sharma, 1972) technique to remove the coverslip, followed by dehydration and mounting in Euparal with a clean coverslip (Grant, 1982b).

Appropriate treatment doses (representing various concentrations and treatment periods) must be carefully selected by range-finding experiments. The duration of the test organism's cell cycle should be kept in mind when selecting appropriate concentrations of the test chemicals, treatment time regimens, and recovery period after treatment. At least 3 treatment doses should be selected for the final testing, with the highest dose determined by phytotoxicity or solubility of the test agent, when possible. The pH of the test solution should be maintained close to neutral with phosphate buffer. Validity of the conclusions is strengthened substantially by demonstration of a dose–response effect. A negative control (water or buffer), solvent control, and a positive control (assuming that such model compounds have been discovered) are required in the experiment. Analysis of at least 100 cells for addition or deletion of a chromosome(s) at metaphase or for a laggard chromosome(s) at anaphase is recommended. The data should be statistically analyzed to evaluate the significance of the results and each experiment repeated to verify the results.

(4.1.2) Leaf spot assay. The leaf spot test system uses leaves of *G. max*, in which leaf color intensity dependents on the gene dosage for the Y_{11} locus. A single Y_{11} gene, as in the heterozygous ($Y_{11}y_{11}$) plant, produces light green color. Two Y_{11} genes ($Y_{11}Y_{11}$) produce dark green color. The $y_{11}y_{11}$ genotype produces almost no chlorophyll. This test system can be used to score what appear to be $Y_{11}Y_{11}y_{11}$ sectors on $Y_{11}y_{11}$ leaves or $Y_{11}Y_{11}Y_{11}$ sectors on $Y_{11}Y_{11}$ leaves. The trisomic condition produces sectors darker in color than the background.

The genetic basis for the origin of spots has been reported (Vig, 1975, 1978). In essence, it is believed that dark green spots on heterozygous leaves result either from homozygosity for gene Y_{11} or from nondisjunction. Homozygosity can result from crossing-over; in that case, the leaves also carry a considerable proportion of twin spots — sectors showing adjacent dark green and yellow spots that are mirror images. The appearance of very dark green spots on $Y_{11}Y_{11}$ leaves is suggested to be caused by 3 doses of gene Y_{11}, indicating nondisjunction. The data can be compiled on a per leaf or leaflet basis for calculation of averages and statistical interpretation.

Any *G. max* variety segregating for the $Y_{11}y_{11}$ genotype can be used for somatic analysis of spots that appear on the leaves as a result of presumptive nondisjunction. Varieties T 219, L 65-1237, and L 72-1937 have been used. Seeds are soaked in a solution of the test chemical; small plants can be exposed to the gaseous or vapor state of the chemical. Embryonic leaves can be coated with lanolin carrying the chemical. Exposure time may vary with concentration, toxicity, or other factors. About 80 seeds or 50 plants is an acceptable population size. Seeds are washed in water to remove excess chemical, sown in galvanized metal flats filled with coarse non-nutritive sand, and watered as necessary. The plants require at least 5% humidity and a temperature of 70°F or higher, and must be grown in an enclosed chamber or greenhouse to avoid insect injury, which can mimic leaf spots.

(4.2) Meiotic cells

The protocol for *H. vulgare* will be described as a model for the detection of induced changes in chromosome number. Because meiotic cells occur only in the inflorescence, plants can be treated at numerous developmental stages prior to initiation of meiosis. The earlier in the plant's development a treatment is administered, the greater the opportunity for diplontic selection to occur, which minimizes the treatment effect measured at meiosis.

Various protocols have been devised to treat plants during different developmental phases ultimately affecting meiotic cells, that can be used conveniently to monitor the kind and magnitude of induced cytogenetic damage. Generally, it is difficult to determine the precise concentration of test agent that is encountered by the target cells (progenitors of the PMC). Seeds can be treated in dormant, pre-soaked, or germinating states with chemical solutions, vapors, gases, or physical agents such as ionizing radiation and ultrasound. Seedlings can be subjected to chronic (usually low dose) or acute (usually high dose) treatment. Chemicals introduced into the root medium are translocated to above ground parts of the plant. Plants can be fumigated (concentrations of the test agent and exposure duration can be precisely controlled (Ma, 1979)), injected with chemical solutions in the immediate environment of the developing inflorescence, or treated with wicks (pads) saturated with the test agent and inserted in the stem near the developing inflorescence. The inflorescence can be sprayed with an atomizer (Ahmed and Grant, 1972) or removed from the plant prior to meiosis and immersed in a solution of the test agent for various time periods. A technique has been developed in which the immature Hordeum inflorescence develops to maturity in culture (Forough-Weir et al., 1976); this allows known concentrations of the test agent to be added to the culture medium for specific time periods.

Most frequently, seeds are chosen for treatment because they are readily available, tolerate a wide range of environmental conditions that can provide insights into the mechanisms involved, can be treated uniformly, permit relatively accurate dosimetry, and are small, allowing large populations to be treated by easily duplicated procedures.

Before the M_0 (treated generation) plant reaches meiosis, the spike is removed, fixed (e.g., in acetic ethanol (1 part glacial acetic acid : 3 parts absolute ethanol)) for up to 24 h, rinsed, and stored in 70% ethanol until analyzed. Differences in the length of the recovery period (time between treatment and the killing/fixation of the inflorescence at meiosis) can affect the outcome.

An anther is sliced and the contents of the anter lobes are squeezed onto a clean, dry microscope slide. 1–2 drops of aceto-carmine (or other appropriate stain such as acetic-orcein) are added, and the preparation is covered with a coverslip, squashed gently with a thumb or a blunt object, and heated slightly. The slide can be analyzed immediately, or the mount can be made semi-permanent by one of several techniques (e.g., the dry ice method described by Conger and Fairchild (1953)). The slide is scanned under low power to locate proper meiotic division figures. Chromosomes are counted under oil immersion.

Usually, more than one slide per spike is prepared to determine intraspike variation. At least 100 cells per spike during diakinesis or metaphase-1 stage are analyzed. In some cases, more than one spike per plant is analyzed.

(5) Discussion

Many short-term bioassays have been developed to evaluate the genotoxicity of environmental chemicals. Most use microbial organisms, transformed animal cells in culture, or laboratory animals; only a few bioassays use plants. It is a common misconception that the toxicological results obtained from studies on microbial organisms or transformed cells in culture may be more relevant to human risk assessment than those from plants. This misconception persists even though the Gene-Tox data base established a 90% concordance between mutagenesis results obtained from animal versus plant systems. The cytogenetic techniques for plant bioassays were developed several decades ago, however, and plant scientists have proposed few new approaches to better use the enormous potential of plants in genetic toxicology research.

The major objective of this literature review was to evaluate the current status of plant test systems

for the detection of chemically induced aneuploidy. Unfortunately, in all plant test systems, an insufficient number of chemicals has been evaluated to warrant judgment. For detecting aneuploidy in somatic cells, *A. cepa, H. vulgare,* and *V. faba* do offer distinct advantages.

Meiotic cells are usually more sensitive indicators of chemically induced aneuploidy than somatic cells. Two test systems that use meiotic cells, *H. vulgare* and *P. americanum,* warrant further attention. Both species have a small number of large chromosomes that makes them easy to work with. *P. americanum* has a very low tolerance for an altered chromosomal complement; monosomics do not survive and trisomics are phenotypically quite distinct from the normal diploid, easily identified by their significantly reduced height and vigor (Dhesi et al., 1983).

To reiterate, plant systems have the advantage of being less expensive than most of the in vitro and in vivo test systems. Vegetative parts, premeiotic, meiotic, and postmeiotic cells can be used and monitored with precise knowledge of cell cycle specificity. Such precision is not easily achieved with meiotic animal cells. The postmeiotic mitosis in the pollen of plants also offers a special assay point not available in animal cells of any species.

(6) Directions for future research

In addition to the traditional squash technique for analysis of chromosomal aberrations, we recommend development of less laborious and more efficient methods to evaluate the ability of chemicals to induce aneuploidy; these may include the use of morphological markers for aneuploidy and plant tissue culture in genetic toxicology studies.

We have briefly described a soybean system that uses leaf spots on an organism in which the intensity of leaf color depends on gene dosage at a specific locus (Vig, 1973). Another promising bioassay uses deficiencies in chlorophyll development in hexaploid wheat seedlings as an indicator of aneuploidy (Rédei et al., 1985). In wheat, recessive alleles at the V locus (short arm of chromosome 3B), when present in two doses, interfere with the greening of the plants at temperatures below 24°C. At higher temperatures, the plants become green after a few leaves have developed, so that sufficient quantities of seeds can be obtained easily.

The v alleles are ineffective in the hemizygous condition (i.e., they behave as recessive suppressors). Since bread wheat is an allohexaploid species, the loss of one allele (chromosome) does not seriously affect the viability of cells monosomic or nullisomic for one particular chromosome, and nondisjunction can be easily scored. The loss of a v-carrying chromosome results in the appearance of a green stripe on an almost white seedling leaf because, in the hemizygous condition, the v allele can no longer suppress leaf pigment production in the cell lineage concerned. After the virescent plants have turned green at a favorable temperature, both products of the nondisjunction are detectable as twin stripes. Three doses of v cause white stripes; a single dose of the allele results in an adjacent dark green stripe on a light green background.

Plant tissue culture technology has made tremendous strides during the last decade. Two different approaches to the use of in vitro culture technology deserve attention. One is the culture of somatic cell populations by (a) long-term continuous growth of roots in defined chemical solutions under controlled environmental conditions (i.e., quality, quantity, and duration of light; temperature; and atmospheric gases) or (b) suspension cultures of cells using the batch method (i.e., periodic subculture into new medium). Another approach is the culture of inflorescences beginning with immature developmental stages. This would circumvent seasonal variability and chemicals could be introduced at known concentrations for specific time periods in the immediate mileu of the PMC. We recognize that cell culture conditions induce chromosomal instability. However, such instability depends on the source of the explants, age and development of the culture, composition of the medium, temperature, and other environmental factors. Species such as *Haplopappus gracilis* and *Crepis capillaris* are more stable under tissue culture conditions than other species and, consequently, may serve as better tools for study of chemically induced aneuploidy. Obviously, the use of these approaches in genetic toxicology has not been developed as yet; the supporting in vitro technology is at hand, and the apparent simplicity of the proposed systems warrant financial support for research and development.

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